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A Wortmannin-Cetuximab As A Double Drug

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Abstract

Double drugs are obtained when two pharmacologically active entities are covalently joined to improve potency. We conjugated the viridin Wm with a self-activating linkage to cetuximab and demonstrated the retention of immunoreactivity by the conjugate. Though cetuximab lacked a growth inhibitory activity against A549 cells, the Wmcetuximab conjugate had an anti-proliferative IC₅₀ of 155 nM in vitro. The chemistry of attaching a self-releasing Wm to clinically approved antibodies is general and, in selected instances, may yield antibody-based double drugs with improved efficacy.

Introduction

Double drugs are obtained when two pharmacologically active entities are covalently joined to obtain a molecule with an improved potency that often results from a change in physical properties of one of the active entities(1, 2). The ability of modified viridins like wortmannin (Wm) to self-activate by generating the active species Wm, offers a mechanism for the design of self-activating viridin double drugs, where X (Figure 1A) is a pharmacologically active entity. We have previously shown that when X is a 70 kDa dextran, a pharmacologically inactive carrier, the resulting self-activating viridin (SAV) prodrug has an improved antiproliferative activity compared to wortmannin (Wm) due to the slow release of active Wm over the 48 h incubation period of the in vitro antiproliferative assay(3). This slow release also occurs in vivo, evident by the nanomolar concentrations of active viridin generated by micromolar concentrations of circulating SAV prodrug(4). The SAV prodrug is anti-inflammatory in animal models of lung inflammation and arthritis(5, 6), as well as being cytostatic in the A549 xenograft model(4).

Cetuximab is a monoclonal antibody that binds to the epidermal growth factor receptor, ErbB1, and generates an antitumor activity through several mechanisms, including an antagonism of growth stimulation by growth factors, and immune mediated mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), and complement-dependent cytotoxicity CDC(7–9). Consistent with a role for immune mediated mechanisms in cetuximab's in vivo activity, cetuximab does not directly inhibit the proliferation of some cultured tumor cell lines, such as the A549 cell line used here(10–12).

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We hypothesized that attaching wortmannin (Wm) to cetuximab using a self-activating linker employed with dextran-based SAV (compound **5a** of the current study) might yield a potentially general conjugation chemistry for the design monoclonal antibody based double-drugs. A key pharmacokinetic property of blood half-life is comparable for the two materials, with human blood half-lives of 60–80 h for a 70 kDa dextran in humans(13, 14) and 112h for cetuximab(15). Thus it appeared that cetuximab, like a 70 kDa dextran, could serve as a reservoir of inactive Wm that slowly self-activated to yield the active species Wm, as we have shown on other occasions(3, 4, 16). We therefore hypothesized that cetuximab might serve as carrier for Wm, enhancing the antibody's antiproliferative activity and acting as a double drug.

Experimental Methods

Reagents

Wortmannin (Wm) was a gift of the natural products branch of the National Cancer Institute. Fluorescein labeled goat anti-human IgG (secondary antibody) was from Beckman Coulter. The NHS ester of 6-fluorescein-5-(and 6)-carboxamidohexanoic acid (FAM) was from Molecular Probes.

Syntheses

The compounds employed are summarized in Figure 1. To obtain the self-activating Wm-cetuximab (**7a**), **2a** was synthesized and converted to the NHS ester of **2a(3)**. To a solution of the NHS ester of **2a** (4 mg, 6 mmol) in DMSO (100 μ L), was added 160 kDa monoclonal antibody cetuximab (3ml, 10mg/ml) in PBS. The solution was stirred and incubated at 37°C for 1.5hr. The conjugate (**7a**) was purified with Sephadex G-50 in a 1 mM phosphate buffer at pH 7.0 followed by lyophilization. The number of moles of Wm per cetuximab were determined by its absorbance at 418 nm. Compound **7b** was prepared from **2b** in a similar fashion. The syntheses of **5a** and **5b**, which use a 70 kDa amino dextran carrier, have been described(3). The moles of Wm attached per mole of carrier were 4.5 (**7a**), 8.8 (**7b**), 7.8 (**5a**) and 13 (**5b**). To obtain the fluorescent compound **8**, cetuximab (1mL, 2 mg) was diluted with 1M sodium bicarbonate buffer (pH 8.3, 100 μ L). 3.91 μ L of 5-FAM (10 mg/mL in DMSO) was added and the mixture was stirred at room temperature for 1 h. The mixture was purified with PD10 column. Antibody concentration was determined by the BCA assay while attached fluorescein was determined from its absorbance at 493 nm. There were 3.19 moles of fluorescein per mole of cetuximab.

Assays

All experiments used A549 cells (ATCC). Cells were maintained in F12-K medium, 10% fetal bovine serum and 1% antibiotics of penicillin-streptomycin at 37°C, 5% CO₂, and 95% humidity. To obtain antibody binding, cells were seeded at a density of 50,000 cells/well in 24-well culture plates and incubated overnight. Dilutions of antibodies in complete media (500 μ L/well) were added. After incubation at 37°C, cells were washed three times with HBSS, detached with trypsin, centrifuged (5 min at 200 $\times$ g), resuspended (200 μ L cold HBSS), and analyzed by flow cytometry (FACSCalibur, Becton-Dickinson). Binding is expressed as the relative cellular fluorescence (RCF), which is the mean fluorescence intensity of treated cells divided by a similar value for untreated cells. To demonstrate immunoreactive cetuximab on the cell surface, cells were treated with cetuximab (37 °C, 10 nM, 1 h) and washed. Then, 500 μ L of fresh media was added to each well and the cells were incubated at 37°C for 0, 1, 2, 3, 5 or 24 h. Cells were then detached by trypsinization, centrifuged, and resuspended in 100 μ L FITC-labeled secondary antibody solution (50:1 dilution in HBSS). After 1 h incubation on ice, cells were washed three times with fresh HBSS and prepared for flow cytometry analysis as described above.

The amount of compound **8** bound per cell at saturation (10 nM) was determined as immunoreactive fluorescein using a fluorescein immunoassay(17). A sulforhodamine B (SRB) dye binding method was performed as an antiproliferative assay(18). Cells were allowed to proliferate for 48 h in the presence of increasing concentrations of drug as shown in Figure 3, where the drug concentration (x-axis) was plotted versus cell mass (y-axis). The IC50 is determined using a four parameter equation (GraphPad Prism software) setting the maximum value as 100% and allowing the fit to generate a minimum non-zero cell mass value (the asymptote at high drug concentrations). Based on the reported doubling time of A549 of 22.9 h (http://dtp.nci.nih.gov/docs/misc/common_files/cell_list.html), complete growth inhibition would yield a cell mass of about 25% of control after the assay's 48 h incubation. The IC50, therefore, is the mid-range point for the change in cell mass (and not the point where cell mass is 50% of control). We term this an “antiproliferative assay” because it is run under conditions allowing cell proliferation, though no direct measurements of cell division were obtained.

Results and Discussion

The self-activation the viridin Wm undergoes when modified at the C20 position by N-methyl hexanoic acid is shown in Figure 1A. An intramolecular attack of the C6 OH generates Wm, while the carboxyl group can be reacted with amines. WmC20 derivatives featuring a secondary amine at C20 (**2a**) and a tertiary amine at C20 (**2b**) were converted to NHS esters and attached to the amino groups of amino-dextran, to yield **5a** and **5b**, or to the amino groups of cetuximab to yield **7a** and **7b**. The compound designations shown in Figure 1B are from our previous communications, with our new cetuximab based compounds designated as **7a**, **7b** and **8**. Thus **2a**, **2b**, **5a** and **5b** are the same as in our earlier publications, with the “a” designation referring to an N(Me) hexanoic acid, self-activating linker reacted with Wm's C20. The “b” designation refers to N(H)hexanoic acid based compounds which do not self-activate due to an extra hydrogen bond stabilization shown in Figure 1B(3, 16).

To determine whether the attachment of Wm to cetuximab (as in **7a** and **7b**) resulted in a reduced immunoreactivity, a self-displacement assay (Figure 2C) was employed. However, we first characterized the binding of the fluorescein labeled **8** to cells as shown in Figures 2A and 2B. Binding of **8** was determined as the relative cell fluorescence (RCF), that is the mean fluorescence intensity determined by FACS of cells incubated with **8** divided the mean fluorescence intensity of control cells. Preliminary experiments indicated the binding of **8** to cells was rapid and independent of incubation times between 1 and 24 hours. We then varied the concentration of **8**, and determined a Kd of 0.40 nM as shown in Figure 2A. The amount of **8** bound per cell at a saturating concentration of 10 nM was determined by a fluorescein hapten immunoassay to be $56,400 \pm 14,200$ molecules per cell. Our estimate of the number of binding sites per cell was about 10 fold higher than Mukohara who used a FACS based bead method(12), while we measured immunoreactive fluorescein from **8** after cell lysis.

With the concentration dependence and number of binding sites for **8** binding to cells in hand, we determined the immunoreactivity of the Wm modified cetuximab **7a** by its ability to displace the binding of **8** (10 nM) from cells (Figure 2B). The similar decrease ($p > 0.05$ for all concentrations) in RCF produced by **7a** and cetuximab indicated the attachment of Wm to cetuximab, as occurred with **7a**, did not impair its immunoreactivity.

A surprising feature of the binding of **8** to A549 cells was lack of time dependence of this binding, which suggested that **8** remained as a stable complex bound to its ErbB1 target receptor on the cell surface. To verify that this was in fact the case, we incubated cells with cetuximab (10 nM, 1 h), washed cells, and detected cell surface cetuximab by the addition of

a fluorescein labeled goat anti-Human IgG. As shown in Figure 3C, the RCF was essentially constant for 6 hours and decreased to 50.6 % of control at 24 h. A 50% decrease in cell fluorescence would be expected at 22.9 hours based on the doubling time of A549 cells, see above. Thus cetuximab appears not to be internalized by A549 cells.

We next assessed the antiproliferative activity of the Wm-cetuximab conjugates (**7a**, **7b**), with the dextran conjugates featuring the same linkages (**5a**, **5b**) serving as non-receptor binding Wm controls. Representative curves for the decrease in cell mass as a function of concentration are shown in Figure 3A and 3B with IC₅₀'s provided in Table 1. Cetuximab had no effect on the proliferation of A549 cells, consistent with the observations of others(12, 19, 20), though cetuximab has been shown to inhibit the growth of A549 xenografts (10, 11), an effect that may reflect immune mediated killing, see above. Based on the similar IC₅₀'s of **5a** and **7a** (Table 1), **5a** and **7a** release Wm into the media in a similar manner (Figures 3C and 3D). Based on the lack of internalization of cetuximab (Figure 2), **7a** was not internalized (Figure 3D).

The antiproliferative activity of cetuximab was greatly enhanced by the attachment of Wm with a self-releasing linkage. That compound, **7a**, exhibited an IC₅₀ of 153 nM on a per mole cetuximab basis (690 nM on a Wm basis at 4.5 Wm/per mole of cetuximab), while cetuximab lacked activity in this assay. This improvement in antiproliferative activity of **7a** relative to cetuximab was not due an immune mediated binding of **7a**, since the IC₅₀ of the self-activating dextran (**5a**) was 700 nM on a wortmannin basis, and not different from the IC₅₀ of **7a** ($p > 0.05$). With both cetuximab and dextran carriers, the configuration at C20 was critical for activity, with non-releasing C20 configurations of **5b** and **7b** showing no antiproliferative activity. The potency of Wm was greatly increased by its attachment to cetuximab with the self-releasing linkage. Wm has an IC₅₀ of 11.4 μ M against A549 cells, while **7a** had an IC₅₀ of 690 nM on a per mole of Wm basis.

There are two conclusions from these studies. First, Wm could be attached to cetuximab at levels that did not affect its immunoreactivity but increased its antiproliferative activity. The experimental approach developed here, attachment of Wm followed by verification of immunoreactivity, could be employed to obtain slowly Wm releasing, immunologically active, monoclonal antibodies. Second, the attachment of Wm to cetuximab increased the antiproliferative activity of both the antibody and Wm. This improvement occurred through the slow release of Wm into culture media, based on the similar antiproliferative IC₅₀ of **5a** and **7a**.

Though we did not demonstrate the immune mediated targeting of Wm to A549 cells in culture, this may still be possible in vivo with appropriate antibody/tumor combinations. The A549 we employed is a Ras driven tumor(21, 22), and the amount of Wm that must targeted to achieve a pharmacological effect might be considerably higher than for PI3 kinase driven tumors, see(23, 24). In addition, our Wm-cetuximab was not internalized by A549 cells, a fate common with cetuximab and other cell lines(25). Internalization of Wm-monoclonal antibodies and receptor recycling might lead to higher intracellular levels of Wm. Finally, a self-releasing Wm antibody might be attached to a monoclonal antibody or protein with an immune suppressive activity, which would then act as a pharmacologically active (but non-targeting) carrier for a slow Wm release. The slow release of Wm from **5a** is immune suppressive in an animal models of lung inflammation and arthritis(5, 6). Wm is a potent antiangiogenic agent(26), offering a mechanism for immune suppression or inhibiting tumor growth without targeting.

From 1980 to 2005 some 206 monoclonal antibodies were studied in clinical trials with 12 being approved(27), offering a wide array of potential Wm-antibody double drugs. The

conjugation of Wm with a self-activating linker to an approved monoclonal antibody offers a potentially general chemistry for the design of double drugs, the pharmacological effectiveness of which will have to be evaluated for each antibody considered.

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Figure 1. Self-activation of WmC20 derivatives and the compounds used

(A) Wm modified at C20 undergoes an intramolecular attack to yield Wm. The attack is independent of the group attached to the carboxyl group of the N(Me)hexanoic acid linker(3). (B) **2a** was converted to an NHS ester and reacted with the amines of cetuximab or amino-dextran(70 kDa) to yield **7a** or **5a**, respectively. Similar reactions were used with the Wm derivative **2b** which features a secondary amine at C20, to yield **5b** or **7b**. “b type” WmC20 derivatives feature a hydrogen bond stabilization and do not self-activate to generate Wm. (C) An NHS ester of a fluorescein hexanoic acid was reacted with cetuximab to yield a fluorescent antibody (**8**).

Figure 2. Binding of FI-cetuximab (8) and Wm-cetuximab (7a) to cells

(A) Concentration dependence of the binding of FI-cetuximab (8) to cells. ($K_d = 0.40$ nM)
 (B) Immunoreactivity of 7a. Cetuximab or 7a blocked the binding of 8 to cells. Data indicating the retention of cetuximab on the cell surface are shown in (C) with the protocol summarized in (D). Cells were exposed to cetuximab for 1 h, washed, and incubated in culture media for varying amounts of time. Immunoreactive cetuximab on the cell surface was detected by a fluorescein labeled goat anti-human IgG.

Figure 3. Increase in Potency for the double drug, Wm-cetuximab

(A) Attaching Wm with a self-releasing linkage to either cetuximab or dextran increased its antiproliferative activity when data are plotted on a mole of Wm basis. (B) Attaching Wm with a self-releasing linker increases Wm potency when data were plotted on a cetuximab basis. (C) Schematic for the activity of Wm-dextran, **5a**. **5a** generates Wm outside of cells, which then enters cells. The slow generation of Wm increases Wm potency, see(3). (D) Schematic for the behavior of Wm-cetuximab, **7a**. Like **5a**, **7a** released Wm outside cells, which then entered cells. **7a** also bound cells and released Wm. The immune-based targeting of **7a** was not a factor in its activity, as the antiproliferative IC₅₀ (Wm basis) was 700 nM for **5a** and 690 nM for **7a**.

Table 1

Antiproliferative Activity Of Wortmannin-Cetuximab Conjugates

Compound	Anti-proliferative IC ₅₀ as Cetuximab, (nM)	Anti-proliferative IC ₅₀ as Wm, (nM)
Cetuximab	Not Detectable*	Not detectable*
7a	155	700±20
7b	>1000	>100000
5a	Not applicable	690 ±20
5b	Not applicable	>100000
Wm	Not applicable	11400±50

Results from three assays on separate occasions are provided.

* Depression of cell mass was too small for an accurate IC₅₀ to be determined.